Telomeres, workload and life-history in great tits

Atema, Els

Chapter 3

Ultra-long telomeres mask attrition of short telomeres in great tits

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Submitted

Abstract

Telomere length (TL) is increasingly used as a biomarker of ageing. TL varies between cells and chromosomes, thus a tissue sample has a TL distribution rather than one TL. The telomere distribution can be divided in shorter (Class II) telomeres and ultra-long (Class III) telomeres (Class I telomeres are static interstitial telomeric sequences). While Class II telomeres typically shorten with age, little is known of Class III telomere dynamics. Given that telomeres shorten at a higher rate in short-lived species, and that within individuals the highest shortening rate is found in the longest telomeres of the Class II distribution, we expected high rates of telomere shortening in great tits, a short-lived species we find to have ultra-long telomeres. Multiple experiments confirmed that the ultra-long telomeres indeed consisted of telomeric repeats rather than sub-telomeric regions. TL averaged over the whole distribution did not significantly change with age. However, more detailed analyses showed that Class II telomeres did shorten with age, whereas Class III telomeres maintained their length. Within the Class II telomere distribution, the longest telomeres shortened faster, like in other species. We conclude that Class II telomeres in great tits are biological most relevant as marker of ageing. Our results suggest there is variation in TL and dynamics between different sets of chromosomes within individuals. This interpretation indicates a need for techniques that measure telomeres for subsets or single chromosomes. Moreover, studies finding no age effects on TL may be confounded by ultra-long telomeres that do not shorten with age.

Introduction

Finding biomarkers of ageing is of wide interest and an increasingly used molecular marker of ageing is telomere length (TL) (e.g. Blackburn *et al.* 2015; Haussmann & Heidinger 2015). Telomeres are non-coding DNA repeats forming the end-caps of linear chromosomes, hereby safeguarding chromosome integrity (Blackburn 1991). TL is to a large extent genetically determined, although heritability estimates vary widely between studies (overview in Atema *et al.* 2015). TL generally shortens with age, although in some studies no age effect was found (overview in Dantzer & Fletcher 2015). Moreover, telomere shortening is accelerated by environmental challenges and the dynamics are related to for instance early life condition (Boonekamp *et al.* 2014; Reichert *et al.* 2014; Watson *et al.* 2015), reproductive effort (Bauch *et al.* 2013), stress during adulthood (Hau *et al.* 2015), diseases (Beirne *et al.* 2014; Asghar *et al.* 2015) and environmental conditions (Angelier *et al.* 2013; Stier *et al.* 2015). Finally, telomeres predict remaining lifespan or survival (e.g. Haussmann *et al.* 2005; Salomons *et al.* 2009; Heidinger *et al.* 2012; Boonekamp *et al.* 2013). Therefore TL, and perhaps in particular telomere shortening, could be used as a read-out parameter of ageing and experienced life-stress.

In birds, telomeric repeats are located at the ends of the chromosomes, but also at interstitial sites in the chromosome (Delany *et al.* 2000; Foote *et al.* 2013). It is unlikely that interstitial telomeres shorten, as terminal telomeres generally do, because this would involve double strand breaks. Hence, as a biomarker of ageing only the terminal telomeres are of interest. Based on their length, two types of telomeres at the end of chromosomes have been described (Delany *et al.* 2000): shorter or Class II telomeres (8-40 kb) which are generally found to shorten with age, and ultra-long or Class III telomeres (up to 2.0 Mb). Delany *et al.* (2000) found no evidence for shortening of Class III telomeres, but her analyses were cross-sectional and data sets were small. Overall, little is known of the dynamics of ultra-long telomeres.

Multiple techniques are available to measure TL and these techniques differ, among other things, in the information they provide, ranging from a point-estimate of average TL in a sample (including interstitial sequences) to the length of single telomeres (Nussey *et al.* 2014). When the aim is to quantify the distribution of TLs at the ends of chromosomes, non-denaturing Terminal Restriction Fragment (TRF) analysis is highly suitable (Haussmann & Vleck 2002). This method yields an image of a smear with a range of TLs with varying optical density, depending on the prevalence of a particular fragment size (Lansdorp *et al.* 1996). Because the DNA is not denatured, the probe binds to the single strand telomere overhang only, and hence interstitial telomeric sequences do not show up on the gel. Using this method, it was shown in jackdaws (Salomons *et al.* 2009) and common terns (Bauch *et al.* 2014) that within individuals the longer telomeres shortened at a higher rate, and predicted survival and other fitness components best. However, these species have negligible ultra-long telomeres (Class III) and hence it is not known whether this finding extends to this Class of telomeres.

To gain an understanding of telomere dynamics across the full size distribution of telomeres, we investigated age dependent patterns in subsets of telomeres in free-living great tits (*Parus major*). In this species we found broad distributions of TLs ranging from 2.1 till >240kb. Following Delany *et al.* (2000) we divided the distribution into Class II (short) telomeres and Class III (ultra-long) telomeres. We analysed age dependent patterns within individuals for the whole distribution of TLs and separately for the different TL classes to identify the best ageing biomarker in this ecological model species.

Materials and methods

Study species and blood sampling

We monitored a population of great tits on Vlieland, an island in the Dutch Wadden Sea, in the years 2011-2015. During the breeding season, adults were caught with spring traps while feeding 8-10 day old nestlings and in winter while roosting in nest boxes (Atema *et al.* 2016). Individuals were identified by their ring number; 70% of the captured birds used in this study had been ringed as nestling. Individuals captured for the first time were ringed and their age (yearling or older) was estimated based on the colour of the wing coverts. Age was known for 101 out of the 105 individuals in our dataset and ranged from 0-7.6 years.

Blood samples were taken from the brachial vein and stored in 2% EDTA at 4-7 °C for up to three weeks. Subsequently, samples were snap-frozen in 40% glycerol buffer and stored at -80 °C.

Telomere terminal restriction fragment analyses

We quantified TL in male great tits using terminal restriction fragment (TRF) assays as described previously (Salomons *et al.* 2009; Atema *et al.* 2015) with some adjustments. In short, DNA from 4 µl of red blood cells was extracted in agarose plugs using the CHEF Mammalian Genomic DNA Plug kit (Bio-Rad Laboratories, Inc., USA). Subsequently DNA from half a plug was digested overnight at 37 °C with a mixture of the restriction enzymes *HindIII* (30 U), *HinfI* (15 U) and *MspI* (30 U) in NEB2 buffer.

The restricted DNA and the ^{32}P end-labelled size standards (1 kb DNA ladder, New England Biolabs, range 0.5-10kb; Molecular Weight Marker XV, Roche Diagnostics, Basel, Switzerland, range 2.4-48.5 kb; NEB MidRange PFG Marker I, New England Biolabs, range 15-242.5 kb) were separated through a 0.8% agarose gel by pulsed field gel electrophoresis at 14 °C for 22 h (4.8 V/cm, initial switch time 1 s, final switch time 25 s). Gels were dried (gel dryer model 538, Bio-Rad Laboratories) and hybridized overnight at 37 °C with ^{32}P-labelled oligonucleotide (5'-CCCTAA-3')$_4$, which bound to the single-stranded overhang of the telomeres. Gels were exposed overnight to a phosphor

screen (PerkinElmer Inc., Waltham, USA), and the radioactive signal was visualized using a phosphor image (Cyclone™ Storage Phosphor System, PerkinElmer) (Fig. 1).

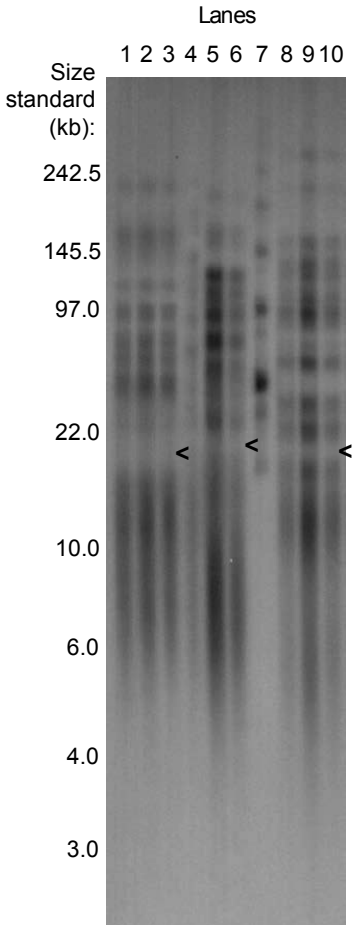

Figure 1. Subset of a non-denatured pulsed-field gel representing the variation in telomere distributions. Represented are repeated samples of three individuals, with the clusters of individuals in lane 1-3, 5-6, 8-10. Lane 4 includes a control sample and lane 7 includes the ultra-long size standard NEB MidRange PFG Marker I. The border between Class II and III telomeres is indicated with black arrows per sample cluster. Note the consistent individual variation in telomere distribution pattern.

We quantified the distribution of TLs based on densitometry using the open-source software IMAGEJ v. 1.38x as described previously (Salomons *et al.* 2009; Atema *et al.* 2015). Our lower molecular weight limit was the point where the optical density was lowest in the region of short telomeres (approximately 3 kb). As upper limit we set the point where the optical density dropped to the background density in the region of long telomeres, which was maximally 240 kb (approximately the limit of our molecular size standards). In rare cases telomeres extended somewhat beyond this upper limit, which we could not quantify.

For each sample we calculated average TL, as well as the TL of every 10th percentile (range 10-90%). Our preliminary results suggested that the short and long telomeres are two different traits reflecting Class II and III telomeres as described by Delany *et al* (2000). Hence, we continued the analyses of age dependent patterns by dividing the distribution of telomeres into two regions: (i) long telomeres, visible as the banded pattern in the high molecular weight region (Class III, approximately 20-240 kb) and (ii) short telomeres, which were visible on the gel as a continuous smear in the short length region (Class II, approximately 3-20 kb). Delany *et al* (2000) suggested that the transition of Class II to Class III telomeres was at 30-40 kb in chicken, without defining a clear border. Based on the optical density plots we defined a border between the end of the short "smear" and the beginning of the long "bands" for each sample individually (range 9-32 kb). This border is visible on the images of the gels as a small gap with a lower optical density between the Class II "smear" and Class III "bands" (Fig. 1). We identified the border by first inspecting the gel image to pinpoint the range in which the border was located, after which we defined the border as the point in that range with the lowest optical density values. This individual border was correlated with age (slope: -211.1 ± 76.4 bp; $F_{1,147.8}$ = 7.63, P = 0.0065, Fig. S1), indicating that the assignment was biologically relevant. However, because there is a slight subjective element in the visually scored threshold we verified results with results obtained with a fixed threshold at 20 kb (see supplementary information S2).

Testing for effects of sub-telomeric regions

Great tit TLs turned out to be extremely long compared with other bird species (see below). A substantial part of the genome consists of repetitive DNA, amongst which are repeats of sequences at sub-telomeric sites (Biscotti *et al.* 2015). Potentially, not all DNA other than telomeric repeats was digested by the 3 restriction enzymes we used which would have left sub-telomeric repeats intact. We therefore carried out multiple experiments to test the hypothesis that the long telomeres we measured can be attributed to sub-telomeric repeats.

First, we applied the exonuclease *Bal31*, an enzyme that preferentially digests double-stranded DNA ends when DNA is intact. DNA was extracted in agarose plugs as described above that we subsequently cut in four equal-sized pieces. These pieces were subjected to different digestion times with *Bal31* (0.1 U in 200 µl of reaction buffer): 0, 20, 80 and 240 minutes. To stop the reaction, plugs were transferred to a tube on ice containing 20 mM Tris, 50 mM EDTA (pH 8.0) buffer. Subsequently plugs were immediately washed three times (10 min each) in EDTA buffer, three times in Tris buffer and placed in 150 µl restriction enzyme reaction buffer for 60 min. Finally, DNA was restricted using the mix with three restriction enzymes and gel-electrophoreses, labelling with the oligonucleotide, and visualization of the radioactive signal were done as described above.

After digestion with *Bal31* there is no single-stranded overhang to label with the telomeric probe and hence DNA had to be denatured for this assay. To this end we followed the procedure as described by Foote *et al.* (2013). Denaturing and subsequent labelling of telomeric sequence will visualize all three Classes of telomeres. The protocol involved denaturing the gels with a buffer (1.5 M NaCl, 0.5 M NaOH) during three times 30 minutes and neutralizing it during two times 30 minutes with a buffer (0.5 M Tris-HCL pH 8.0, 1.5 M NaCl) at room temperature. Gels were hybridized with the ^{32}P-labelled oligonucleotide (5'-CCCTAA-3')$_4$ and the radioactive signal was visualized following the same protocol as used with the non-denatured gels. Because labelling now occurred along the full length of telomeric repeats, differences in the banding pattern in the long region of the telomere distribution were visualised with higher contrast, allowing for a more detailed comparison of different treatments.

Second, we used a mixture with 7 restriction enzymes, instead of our standard 3 restriction enzymes, to increase the likelihood that all DNA other than telomeric sequence was digested. The restriction enzymes we added to our standard set were *HphI* (15 U), *MnlI* (15 U), *RsaI* (15 U) and *HaeIII* (15 U) (New England Biolabs, Inc., Beverly MS, USA). Gel-electrophoreses, labelling with the oligonucleotide, and visualization of the radioactive signal were done as described above, including denaturation after the normal labelling protocol.

Third, we measured telomeres using qPCR in great tits, to be compared with blue tits (*Cyanistes caeruleus*, also sampled on Vlieland) of which we also obtained TRF measurements of the same set of samples. The average TL measured with TRF is half as long in blue tits compared to great tits (see below). Finding a similar ratio with qPCR, which we can safely assume to measure exclusively telomeric repeats, would further support our results found with TRF. As a control gene we used GAPDH, amplified with primers we previously designed for great- and blue tits (Atema *et al.* 2013). The qPCR reaction was executed as previously described (Atema *et al.* 2013), with small adjustments. DNA samples were diluted to a concentration of 0.83 ng/µl, each reaction contained 5 ng of DNA. The reaction for telomere (500 nM for forward and reverse primer) and GAPDH (100 nM for forward and reverse primer) was done on separate plates, with DNA of great- and blue tits on the same plate. We calculated the T/S ratio as:

$$\frac{Eff_{telo}\,\hat{}\,\Delta Cq_{telo}}{Eff_{GAPDH}\,\hat{}\,\Delta Cq_{GAPDH}} \qquad \text{(Pfaffl 2001)}$$

We calculated the mean amplification efficiency (Eff) and cycle quantification (Cq) values with the programme LinRegPCR (version 12.13; Ruijter *et al.* 2009). ΔCq was calculated as the Cq values of the control subtracted by the Cq value of the sample. Mean Eff_{telo} was 1.94 ± 0.014 and mean Eff_{GAPDH} was 2.11 ± 0.0092, both close to the expected efficiency of 2.

Statistics

We tested for effects of age on TL using linear mixed models on a longitudinal dataset (2 individuals sampled 6 times, 4 individuals sampled 4 times, 32 individuals sampled 3 times, 60 individuals sampled 2 times and 7 individuals sampled once), including individual identity and gel identity as random effects.

Given that we found hints for telomere elongation in a part of our dataset, we tested whether telomere elongation was real or could be attributed to measurement error following Simons *et al.* (2014).

Analyses were done in JMP 7.0 and R 3.0.2 (R Development Core Team 2008). Unless mentioned otherwise, estimates are presented as average ± standard error (s.e.).

Results

Full telomere distribution

The distribution of TLs included extremely long telomeres, exceeding 240 kb in some cases. The average length of telomeres was 47.1 ± 2.0 kb (N = 251), which is more than twice as long as found previously in other bird species analysed in our lab (Fig. 2), and about seven times as long as telomeres of human adults (e.g. Verhulst *et al.* 2013). We restricted this comparison to species measured in our lab, to minimize methodological effects on TL.

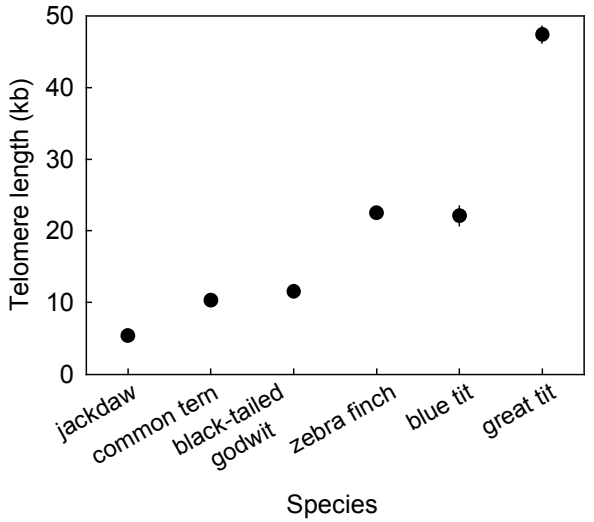

Figure 2. Population mean TLs in adults of different bird species as measured in our lab with TRF analysis in jackdaw (Salomons *et al.* 2009), common tern (Bauch et al. 2014), black-tailed godwit (Atema *et al.* 2011), zebra finch (Atema *et al.* 2015), blue- and great tit (this study). Error bars that are not visible are smaller than the marker.

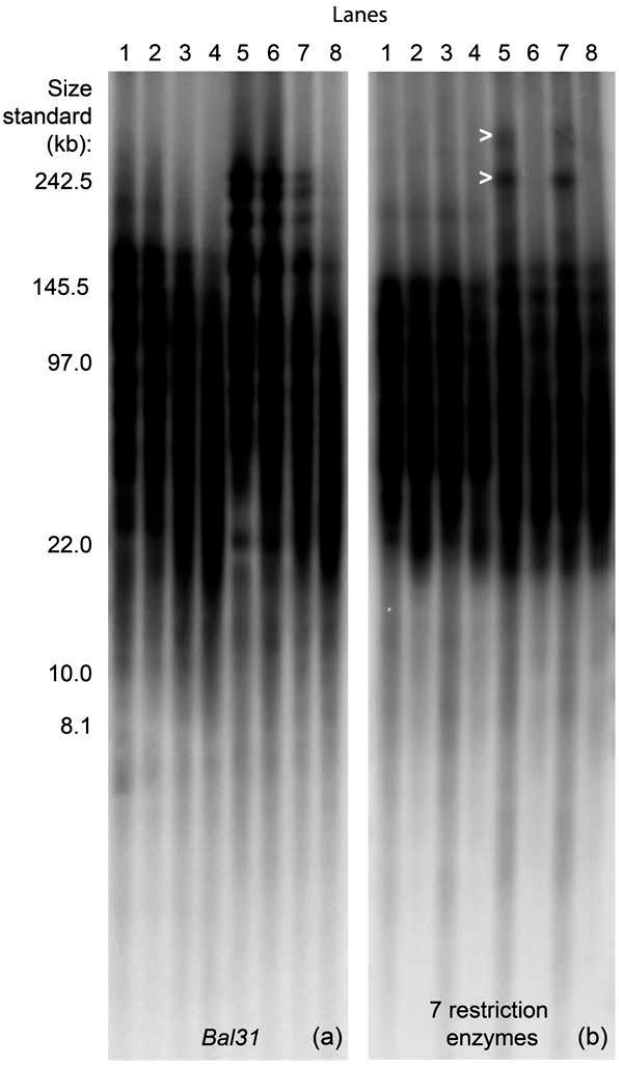

Figure 3. Tests to ensure we quantified exclusively end-telomeric repeats. (a) The telomere distribution as visible after digestion of double-stranded DNA ends with *Bal 31*. DNA was digested over a gradient of time (0, 20, 80 and 240 seconds) as shown in two individuals (lanes 1-4 and 5-8). (b) The telomere distribution as visible after restriction with 3 (odd lanes) or 7 (even lanes) restriction enzymes. The test was run in two individuals (lanes 1-4 and 5-8) and in each individual at two different ages (age 1: lanes 1, 2 and 5, 6; age 2: lanes 3, 4 and 7, 8). The difference caused by the additional restriction enzymes, situated in the extremely large region of the distribution, is depicted with white arrows.

Ultra-long telomeres or sub-telomeric repeats?

The ultra-long TLs we measured could be due to sub-telomeric repeats in the flanking regions of the telomeres, rather than containing exclusively telomeric repeats. We ran three experiments to test whether we quantified exclusively telomeric repeats.

Bal31: Over the gradient of digestion times, the signal in the larger weight region reduced and there was a shift in the distribution of telomeres towards the lower weight region in which the signal became more intense (Fig. 3a). This indicates that the restriction fragments consist largely of telomeric sequences, because otherwise the signal would have weakened more and shifted less. The average TL estimated after 240 minutes of digestion with *Bal31* in 5 different samples was reduced by 32% compared to the start of the digestion procedure.

7 Restriction enzymes: We compared TL of 2 individuals (2 samples each at different ages) with mixes containing 3 (used throughout our study) and 7 restriction enzymes. The only notable difference from visual inspection of the distribution was the disappearance of a band outside our measurement range (i.e. >240 kb, indicated in Fig. 3b), suggesting that only this extremely long telomeric repeat was confounded by sub-telomeric repeats. The additional restriction enzymes on average caused a reduction of TL of 2.8 kb, which is consistent with a decrease of 5.8%. Since we found no substantial differences compared to the use of three restriction enzymes this indicates that our measurements were not confounded by sub-telomeric repeats in the flanking regions.

qPCR: We compared TLs quantified with TRF and qPCR in the same samples. Independent of technique, TL*s* were twice as long in great tits compared to blue tits (ratio blue tit:great tit TRF = 1:2.02; qPCR 1:2.16; Fig. 4). This finding further indicates that the ultra-long telomeres as quantified by the TRF assay represented telomeres rather than sub-telomeric regions.

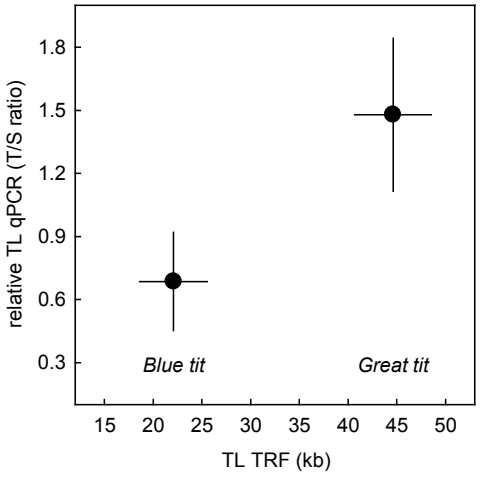

Figure 4. Average TLs (± s.e.) quantified with TRF assays (x-axis) and relative qPCR (y-axis) in blue- and great tits.

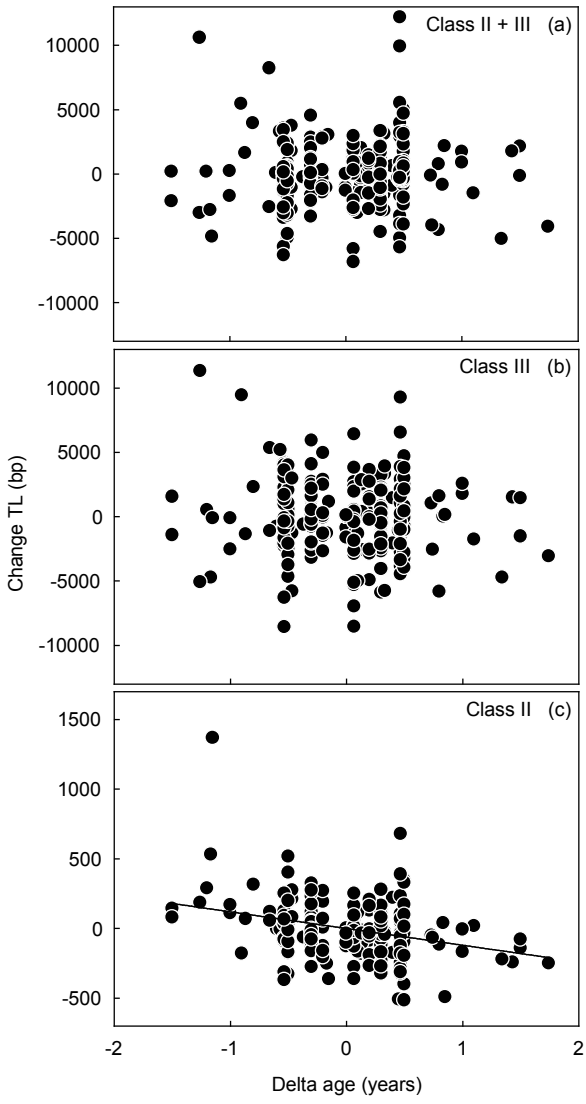

Figure 5. Change of TL (bp) in the full (a), long Class III (b) and short Class II (c) telomere distribution plotted against delta age (years). TL did not significantly decline with age in the complete sample and Class III (P>0.50), while Class II telomeres shortened significantly with age. In the analysis individual identity was controlled for (random effect) and to let the figure reflect the analysis the data on both axis were expressed as deviations from the individual mean age and telomere length calculated over the different samples per individual. Note substantially smaller scale on y-axis of bottom panel.

Full telomere distribution: effect of age

Individual variation (random effect) in the average TL of the full distribution of telomeres explained 51.2% of the total variation. We found no effects of age on the average TL calculated over the full distribution (slope: 213.5 ± 335.1; $F_{1,218.8}$ = 0.41, P = 0.52; Fig. 5a). TL at the 10th percentile was 6.0 ± 0.12 kb, whereas TL at the 90th percentile was 134.8 ± 4.6 kb (Table 1). We found no significant effect of age on TL at the any of the nine percentiles (P: 0.31-0.88).

In contrast to what could be expected based on findings in other bird species, the data suggested that on average telomeres in the long region of the distribution (percentile 50-90, Table 1) elongated with age. We tested whether there was elongation using the statistical approach proposed by Simons et al. (2014) to detect whether in part of the sample there is significant elongation but could not reject the null hypothesis that Class III telomeres did not elongate ($F_{16,37}$ = 1.46, P = 0.17).

However, in the range representing short telomeres (10-30%) we found hints for the expected telomere shortening with age (Table 1). In subsequent analyses we therefore analysed the ultra-long Class III and short Class II telomere distributions separately.

Table 1. Estimates (s.e.), F ratio and P-value from mixed models testing for effects of age on TL across the percentiles in the full distribution.

Parameter TL	Estimate (s.e.)	F ratio	P-value
average	213.5 (335.1)	0.41	0.52
percentile:			
10	-11.5 (13.5)	0.73	0.40
20	-12.9 (30.8)	0.18	0.67
30	-37.5 (83.0)	0.20	0.65
40	29.9 (205.0)	0.021	0.88
50	328.9 (322.9)	1.03	0.31
60	406.0 (446.3)	0.83	0.36
70	244.3 (617.5)	0.16	0.69
80	441.3 (802.5)	0.30	0.58
90	348.3 (828.9)	0.18	0.67

Class III telomeres

The average TL of the Class III telomeres was 83.6 ± 2.0 kb. Individual variation in the average TL in this region of the distribution of TRF assays accounted for 73.3% of the total variation. Despite the longitudinal character of our dataset, we found little evidence

for an age effect on TL in the Class III telomeres (slope: -73.6 bp/yr ± 428.1; $F_{1,241}$ = 0.029, P = 0.86; Fig. 5b).

We quantified TL at percentiles 10-90% within the Class III TL distribution. Although the estimates of the change of TL with age were negative up to the 80th percentile, we found no significant changes of TL with age across the distribution of Class III telomeres (Fig. 6).

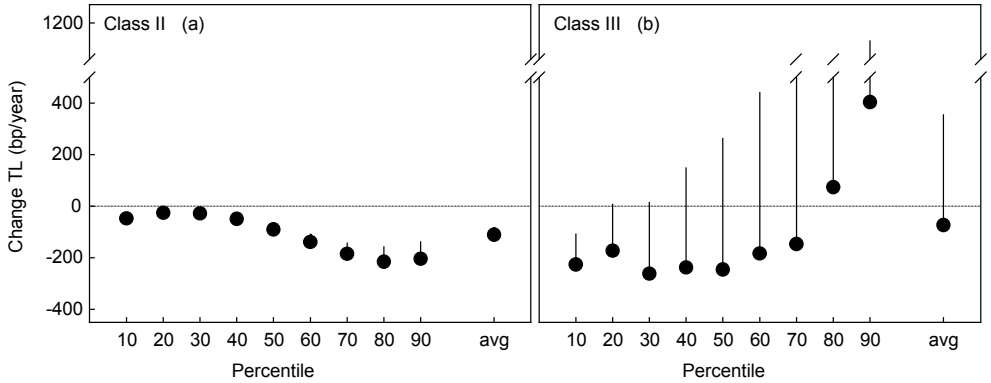

Figure 6. Telomere shortening rate (± s.e.) at different TL percentiles as estimated with the mixed model (bp/year). (a) Class II TL significantly shortened across all percentiles (P: >0.0001-0.0092). (b) No significant shortening across the Class III TL percentiles (10th percentile P = 0.055; other percentiles P: 0.34-0.92). Note increase in s.e. with percentile, which is due to the logarithmic nature of the gels. Furthermore, the rate of shortening in Class II telomeres was higher compared to the shortening rates in the 10th-30th percentiles in the analyses of the full distribution (table 1). This is due to the fact that the 30th percentile of the full distribution in some cases will include Class III telomeres, causing underestimation of telomere shortening.

Class II telomeres

The average TL of the Class II telomeres was 8.7 ± 0.2 kb. Individual variation (random effect) in average Class II TL accounted for 80.5% of the total variation (Fig. 7). Class II TL shortened significantly with age (slope: -111.2 ± 27.1 bp/year; $F_{1,155.1}$ = 16.78, P < 0.0001; Fig. 5c).

When quantifying TL at percentiles 10-90% within the Class II TL distribution we found significant shortening of TL with age at all percentiles (all P ≤ 0.0092). More importantly, because in agreement with our findings in other species, higher percentiles within the Class II TL distribution lost more base pairs per year than the lower percentiles (Fig. 6).

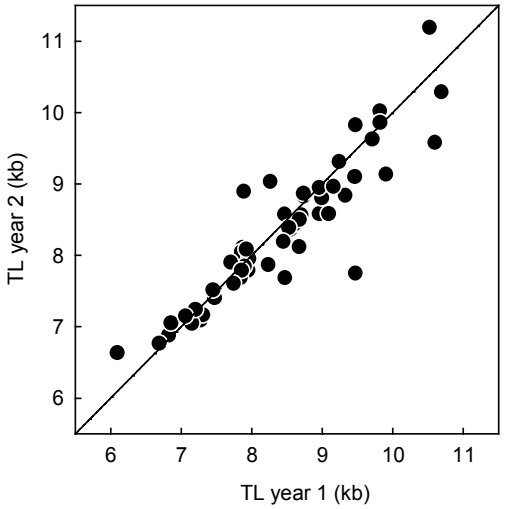

Figure 7. Class II TL in year 2 plotted against TL in year 1 (N = 53 individuals). The dotted line shows where subsequent measurements are equal (y=x). All dots which fall below that line are individuals in which TL was shorter when recaptured 1 year later. For graphical purpose we only plotted individuals with two samples with 1 year interval; in the analyses we included all measurements.

Discussion

TL typically varies between species (Haussmann *et al.* 2003; Lorenzini *et al.* 2009) and we found great tits to have very long telomeres compared to humans and other bird species. Long sub-telomeric regions, not digested by our standard set of three restriction enzymes, could potentially explain this finding. However, results of three different experiments make this explanation unlikely, and we therefore conclude that great tits have long TLs compared to other bird species. TL measured with TRF in samples from great tits in Wytham woods showed similar long telomeric patterns as found in our Vlieland population (Mulder, Bouwhuis *et al*. unpublished observations), suggesting that ultra-long telomeres are a general feature of great tits. In mammals, TL variation was in part explained by body size, with small mammal species having longer telomeres (Lorenzini *et al.* 2009). In the small set of species for which we could compare TL (Fig. 1), there is also a trend that smaller and shorter lived species have longer telomeres, but clearly a much larger species set is required to analyse interspecific variation in avian TL.

Ultra-long telomeres were previously found in several mammal species. For example, Kipling & Cooke (1990) found telomeres up to 140 kb in laboratory mice that did not shorten with age in a cross-sectional analysis. Zhdanova *et al.* (2010) found TL

up to 300 kb in wild Iberian shrews, and Wistar rats have telomeres up to 100 kb (Cherif *et al.* 2003). Surprisingly, telomeres in wild mice are considerably shorter, suggesting that selection for long telomeres somehow took place in laboratory bred mice and rats (reviewed in Eisenberg 2011). Domestication however does not explain the existence of ultra-long telomeres in wild great tits or Iberian shrews.

The ultra-long telomeres were due to high prevalence of Class III telomeres, which have previously been demonstrated in chickens, several raptor species and cranes (Delany *et al.* 2000). Why Class III telomeres are so numerous in great tits is a question to which we can only give speculative answer. Nanda *et al.* (2002) compared the distribution of telomeric repeats at the chromosome level in 16 bird species using FISH and found micro-chromosomes to display a large number of telomere sequence. This is consistent with the finding of Delany *et al.* (2000) that Class III telomeres were more numerous in species with more micro-chromosomes. Great tits were estimated to have 40 different chromosomes (2n = 80) of which about half were classified as micro-chromosomes (Nanda *et al.* 2011; van Oers *et al.* 2014). Thus the large number of micro-chromosomes might, at least partly, explain the high prevalence of ultra-long telomeres in great tits. It is worth noting that this explanation contrasts with the positive correlation between chromosome size and telomere length observed in the human genome (Wise *et al.* 2009). On the other hand, the smallest human chromosome (#21) is larger than all great tit micro-chromosomes (Santure *et al.* 2013), and different processes may act on different size ranges.

Telomere dynamics

Following Delany *et al.* (2000) we separated the telomeric distribution in two different Classes (II and III). Class III telomeres did not shorten with age, which explains why TL calculated over the full distribution did not change with age. This was unexpected, given that within individual birds and humans the longer telomeres lose more base pairs than the shorter telomeres (Kimura *et al.* 2007; Salomons *et al.* 2009; Bauch *et al.* 2014). In theory, the finding that Class III telomeres do not shorten with age could be due to insufficient statistical power, and we can of course not exclude that a small age effect would be detectable in a much larger data set. However, we anticipate that such an (as yet undetected) effect would be small, given that (i) the precision of our measurements was high, (ii) due to our method, we included only terminal telomeres in our measurements (interstitial telomeric sequences do not shorten with age), and (iii) we had a large set of longitudinal measurements that yields substantially more statistical power than cross-sectional data, due to large TL variation between individuals.

In contrast to Class III telomeres, Class II TL did shorten with age. Based on the relationship between maximum lifespan and telomere shortening in other bird species, we would expect a shortening rate between 50 and 250 bp per year in great tits (Dantzer & Fletcher 2015). The observed rate of telomere shortening of Class II

telomeres was 111 bp/year, which falls well within the expected range. Moreover, we found the higher telomere loss rates at the longer percentiles of the Class II telomere distribution, as previously shown using the same approach in jackdaws (Salomons *et al.* 2009) and common terns (Bauch *et al.* 2014). Because of the similar dynamics as found in other species, we suggest that Class II telomeres might be indicators for biological age, which should be further tested in relation to for instance survival. The distribution of Class II telomeres in great tits overlaps with the telomere distribution in other species with few Class III telomeres such as jackdaws and common terns. It is interesting to note therefore that great tit Class II telomere dynamics resembles the dynamics of these same species, whereas Class III telomeres do not.

The border to distinguish between Class II and III telomeres was not clearly set by Delany et al. (2000), and visual inspection of their gel images suggests the border position to differ between species. Fortunately, Class II and Class III telomeres could be defined relatively easy in great tits by the gap in optical density distribution between the two Classes (Fig. 1). This distinction is for instance less obvious in zebra finches (see gel images Criscuolo *et al.* 2009; Foote *et al.* 2013). We found that the border value consistently varied between individuals and decreased with age, indicating that defining the border position for each individual smear is of importance for estimating TL separately for TL Classes II and III. However, this also introduces a risk of biased scoring, in particular when the border is more difficult to determine than in great tits. It is encouraging therefore that when we estimated TL from our gels using a fixed border at 20 kb, the same pattern emerged, with the telomeres <20 kb shortening with age, while no such effect was found in telomeres >20 kb.

Methodological implications

Our findings indicate that there are sets of chromosomes within the great tit genome that differ strongly in the size of telomeres and rate at which they change in length, and that in particular the shorter (Class II) telomeres within the genome show a pattern that is consistent with markers of ageing. Being able to identify the shorter telomeres within the genome depends on the telomere measurement technique that is employed. Some techniques yield a single estimate related to the number of telomere repeats throughout the genome (e.g. qPCR, dotblot), whereas other techniques (e.g. TRF, Q-FISH) yield a telomere length distribution for each sample, representing variation in telomere length between cells and chromosomes (Nussey *et al.* 2014). In our study, we would have concluded that TL is independent of age when we would have used one of the techniques that yield a single estimate to characterize telomere length. Likewise, it seems possible that other studies that concluded that TL was independent of age would have reached a different conclusion when a more informative technique had been employed. For instance no effect of age on TL estimated with qPCR was found in leatherback turtles (Plot *et al.* 2012), which could be due to the presence of Class III

telomeres, given that turtles also have micro-chromosomes (Ellegren 2013). Hence we recommend investigating the TL distribution with a suitable technique (usually TRF) before investing in techniques that yield only a single TL estimate, and resort to a technique yielding more detailed information when ultra-long telomeres are numerous.

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Supplementary information

S1. Relation individual border and age

There is substantial variation between individuals in the pattern of the telomere distribution. Hence we started our analyses by defining individual borders between the end of the short "smear" and the beginning of the long "bands". This individual border correlated highly with age ($F_{1,147.8}$ = 7.63, P = 0.0065; Fig. S1), indicating biological relevance of these estimates.

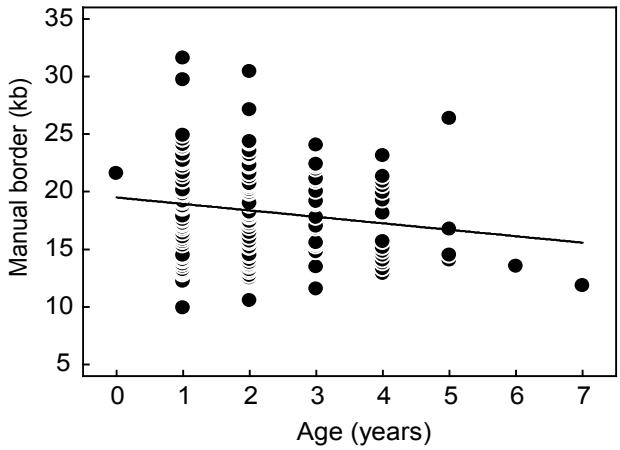

Figure S1. Correlation between the TLs at which we assigned the individual border and age for the individual samples.

S2. Comparing individual border with 20 kb border

To check for robustness of the estimate of TLs using an individual border to distinguish between telomere Classes, we repeated the analysis of age effects on TL estimated with a fixed border of 20 kb. The average of the individual border we used in our study is 18.6 kb. By applying a border slightly above this average, that is a border of 20 kb, we cut off fewer telomere sequences in the distribution of the short smear. Both estimates of Class II TL (individual border and border of 20 kb) correlated reasonably well in most cases (r^2 = 0.41; Fig. S2) but there are also some clear exceptions pointing out the value of individually set borders.

With a border of 20 kb the average TL of the Class III telomeres was 85.4 ± 2.4. Individual variation (random effect) in the average TL in this region of the distribution of TRF assays accounted for 60.9% of the total variation. We found no effects of age on the change of Class III TL ($F_{1,241}$ = 0.13, P = 0.72).

With a fixed border of 20 kb the average TL of the Class II telomeres was 8.9 ± 0.1 kb. Individual variation in the average TL in this region of the distribution of TRF assays accounted for 79.2% of the total variation. TL shortened significantly with age in this part of the telomere distribution ($F_{1,155.1}$ = 10.29, P < 0.0016; Fig. S3). The rate of shortening was 63.8 ± 19.9 bp/year, clearly below the value of 111 bp/year we found with the individually set border. We divided the distribution of short telomeres in percentiles ranging from 10-90% (Fig. S4). We found significant shortening of TL with age in all percentiles. The largest telomere shortening was found in the higher percentiles, thus the longer Class II telomeres.

To summarize, when using a fixed border of 20 kb the findings are similar to our results presented in the paper. However the estimate of Class II shortening rate was smaller when using a fixed border, implying that this approach underestimates telomere shortening rate. Hence we recommend setting the border between Class II and III telomeres in great tits individually.

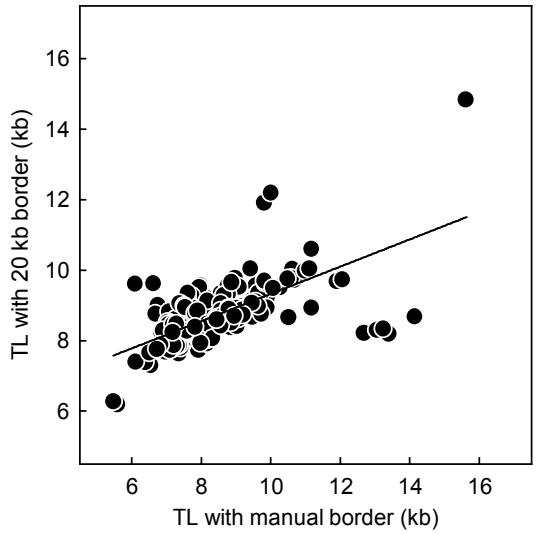

Figure S2. The correlation between Class II TLs estimated with a fixed upper border of 20 kb and Class II TLs estimated with an individual upper border set for each sample.

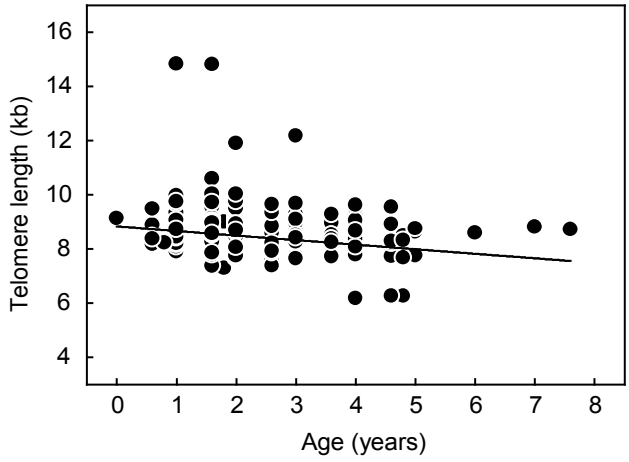

Figure S3. Class II TL (kb) plotted against age (years). In the analyses we corrected for repeated measurements by including individual identity as random effect.

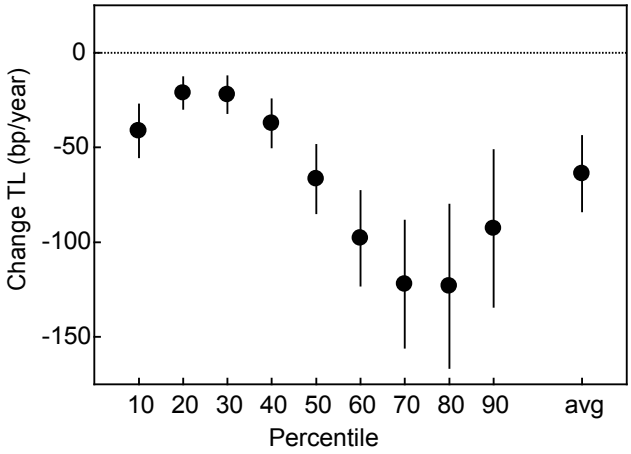

Figure S4. Telomere shortening rate of different Class II percentiles (measured with a fixed Class II / III border at 20 kb) and the average shortening of Class II telomeres as estimated with the mixed model (bp/year).

PART II

Genes, workload and life-history